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Molecular Determinants for Cyclic Nucleotide Binding to the Regulatory Domains of Phosphodiesterase 2A*

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Binding of cGMP to the GAF-B domain of phosphodiesterase 2A allosterically activates catalytic activity. We report here a series of mutagenesis studies on the GAF-B domain of PDE2A that support a novel mechanism for molecular recognition of cGMP. Alanine mutations of Phe-438, Asp-439, and Thr-488, amino acids that interact with the pyrimidine ring, decrease cGMP affinity slightly but increase cAMP affinity by up to 8-fold. Each interaction is required to provide for cAMP/cGMP specificity. Mutations of any of the residues that interact with the phosphate-ribose moiety or the imidazole ring abolish cGMP binding. Thus, residues that interact with the pyrimidine ring collectively control cAMP/cGMP specificity, whereas residues that bind the phosphate-ribose moiety and imidazole ring are critical for high affinity binding. Similar decreases in binding were found for mutations made in a bacterially expressed GAF-A/B plus catalytic domain construct. Because these constructs had very high catalytic activity, it appears that these mutations did not cause a global denaturation. The affinities of cAMP and cGMP for wild-type GAF-B alone were ~4-fold greater than for the holoenzyme, suggesting that the presence of neighboring domains alters the conformation of GAF-B. More importantly, the PDE2A GAF-B, GAF-A/B, GAF-A/B+C domains, and holoenzyme all bind cGMP with much higher affinity than has previously been reported. This high affinity suggests that cGMP binding to PDE2 GAF-B activates the enzyme rapidly, stoichiometrically, and in an all or none fashion, rather than variably over a large range of cyclic nucleotide concentrations.

Eleven different phosphodiesterase (PDE)[1] families, each containing multiple genes, have been described and differ in substrate specificity, regulatory properties, inhibitor profiles, and tissue distribution (1). The cGMP-stimulated PDEs (PDE2s) are one of three families of PDEs (PDE2s, PDE5s, and PDE6s) that are confirmed to contain allosteric cyclic nucleotide binding sites that bind cGMP (2). Two other cGMP PDE families, PDE10A and PDE11A, have homologous regulatory segments (3, 4). In PDE2A, cGMP binding to an allosteric site stimulates catalytic activity, which has numerous physiological consequences *in vivo*. For example, atrial natriuretic peptide stimulation of cGMP production and subsequent activation of PDE2A in the adrenal cortex decreases aldosterone secretion, thereby possibly mediating the effect of the hormone on fluid volume (5).

Previous studies have shown that 1 mol of cGMP is bound per PDE2 monomer (6). Each PDE2A monomer contains a tandem pair of domains (GAF-A and GAF-B) that are now known to be part of a large family of small molecule binding domains called GAF domains (Fig. 1) (7, 8). GAF domains were first recognized by sequence homology in cGMP-specific PDEs, bacterial adenylyl cyclases, and FhlA, a bacterial transcription modulator; they have now been shown to exist in over 1360 different proteins in organisms ranging from humans to sponges (7, 8). Cyclic GMP appears only to bind GAF-B in PDE2A (9) and GAF-A in PDE5A (10). Recently, a mammalian PDE2 GAF domain has been shown capable of activating the adenylyl cyclase cyaB1 of *Anabaena*, a species of cyanobacterium, demonstrating that the function of these domains has been conserved in species separated by over 2 billion years of evolution (11).

The GAF domains of cGMP-regulated PDEs and *Anabaena* adenylyl cyclase cyaB1 are distinguished from most other GAF domains by the presence of a conserved NKX_nFX_3DE (NKFDE) motif (12). The NKFDE motif is conserved in almost all PDE GAF domains and has been proposed to be involved directly in cGMP binding (13–15). In the bovine PDE5A holoenzyme, substitution of Asn-276, Lys-277, or Asp-289 with alanine in the NKFDE motif of the GAF-A domain has been reported to increase the apparent K_D for cGMP from 1.3 μM to between 12 and 60 μM (13). In the human PDE5A GAF-A domain expressed alone, however, mutation of the aspartate in the NKFDE motif to alanine (D299A) only weakened binding by 3-fold from 27 to 78 nM (16). The high affinity binding for the human isoform was attributed to the absence of the GAF-B and N-terminal domains of PDE5A in the expression construct (16).

In the crystal structure of PDE2A GAF-A/B, the NKFDE motif residues of GAF-B are not in contact with cGMP (9). On the contrary, they are at the other side of a β sheet making up the floor of the cGMP binding pocket of the GAF-B domain, far from the cGMP binding site (Fig. 2*A*) (9). The crystal structure of PDE2A GAF-A/B also shows continuous electron density between the lysine and aspartate in this motif, which may indicate that a salt bridge forms between these residues (9).

The ability of cAMP- and cGMP-dependent kinases, cyclic nucleotide-gated channels, and cyclic nucleotide phosphodiesterases to discriminate between cAMP and cGMP is an integral part of how these signaling pathways react to cellular stimuli. A single amino acid has been shown to be critical in controlling the specificity of cyclic nucleotide binding to cAMP- and cGMP-dependent kinases, cyclic nucleotide gated channels, and adenylyl and guanylyl cyclases (17–19). Many high affinity gua-

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[1] The abbreviations used are: PDE, 3′:5′-cyclic nucleotide phosphodiesterase; PDE2, cGMP-stimulated phosphodiesterase; GAF, cGMP-regulated PDEs, *Anabaena* adenylyl cyclase, *E. coli* protein FhlA.

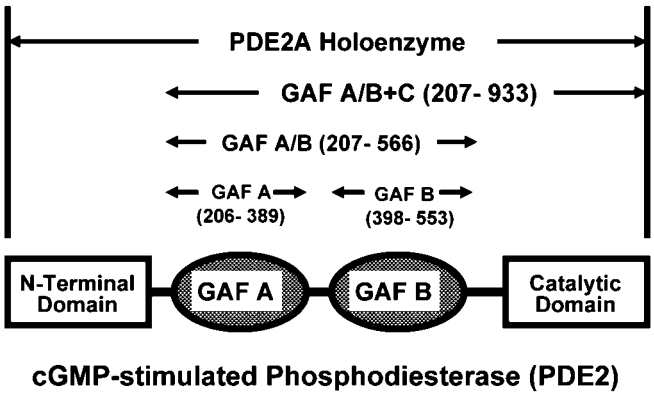

FIG. 1. **PDE2A holoenzyme and GAF domain proteins.** Schematic diagram showing the various constructs generated in this study. The *numbers in parentheses* are the residue boundaries of the individual PDE2A GAF domain constructs.

nine nucleotide-binding proteins utilize the carboxylate group of an aspartate or glutamate residue to bind both the N-1 and N-2 of cGMP (17, 20–23). However, there have been relatively few studies probing these details in PDEs. In one study, mutagenesis of Asp-289 within the cGMP binding GAF domain of PDE5A was shown to influence cAMP/cGMP specificity when the pH was lowered from 9.5 to 5.2 (24). Otherwise, little is known about the molecular determinants of substrate specificity for any GAF domain-containing protein.

The amino acid residues in close contact with the nucleotide have been revealed in the crystal structures of several nucleotide-binding proteins in complex with substrates or analogs (25–28). However, it also is known that the crystal structure of a complex often does not adequately characterize the relative importance of each close residue for nucleotide binding or nucleotide discrimination. In the present study, we used the recently determined 2.9-Å crystal structure of PDE2A GAF-A/B bound to cGMP (9) as a guide for mutagenesis to determine to what extent various residues lining the GAF-B domain pocket determine cAMP and cGMP binding affinity and specificity.

EXPERIMENTAL PROCEDURES

Materials—[8-^{3}H]cGMP and [5,8-^{3}H]cAMP were purchased from PerkinElmer Life Sciences. cAMP (sodium salt), cGMP (sodium salt), epoxy-activated Sepharose 6B, pepstatin A, phenylmethylsulfonyl fluoride, dithiothreitol, and isopropyl-β-D-thiogalactopyranoside were obtained from Sigma.

Cloning and Site-directed Mutagenesis—cDNA for mouse PDE2A2 GAF-A (residues 206–386), GAF-B (residues 398–553), GAF-A/B plus catalytic domain (GAF-A/B+C) (residues 207–933) were cloned into a derivative of the pMW172 vector (Fig. 1) (29). GAF-A/B (residues 207–566) had previously been cloned into the same vector (9). The vector-derived sequence LE(H6) was appended to the C terminus, and a Met to the N terminus. The QuikChange site-directed mutagenesis kit (Stratagene) was used to make point mutations in the pMW172 clone according to the protocol from Stratagene. Mutagenic oligonucleotides (see Table I) were ordered from Integrated DNA Technologies (Coralville, IA). *Escherichia coli* XL-1 blue cells were used for all DNA manipulations. DNA was purified from small scale vector preparations using a Qiagen Plasmid Mini kit according to the manufacturer's protocol. DNA segments subjected to mutagenesis and subcloning reactions were sequenced in their entirety to ensure the presence of the desired mutation and proper in-frame subcloning. The same procedures and oligonucleotides were used to make the PDE2A GAF-A/B plus catalytic domain constructs (A/B+C).

Expression of Wild Type and Mutant PDE2 GAF Domains—Wild-type and mutant GAF domains were expressed in C41 (30), a derivative of the *E. coli* strain BL21(DE3). The mutants were expressed at levels comparable to that of the wild-type GAF domains (5–7 mg/liter of culture). Luria broth with 50 μg/ml ampicillin was used, and cells were first grown at 37 °C until A_{600} = 0.5. The cells were then induced with isopropyl-β-D-thiogalactopyranoside (30 mg/liter) and incubated at 16 °C for 22 h. Cells were disrupted in lysis buffer (100 mM NaCl, 20 mM Tris, pH 7.5, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 1 mM β-mercaptoethanol) by microfluidization (10,000 p.s.i.) (Microfluidics, Newton, MA) (31), and then centrifuged at 16,000 × *g* for 15 min. The supernatant was filtered through Whatman filter paper and then chromatographed through an epoxy-Sepharose cGMP affinity column (32). After elution with 1 mM cGMP (200 mM NaCl, 20 mM Tris, pH 7.5, 0.1 mM EDTA, and 1 mM β-mercaptoethanol) and concentration using a Centriplus centrifugal filter (Amicon), aggregated protein was removed by gel filtration on Superose 12 (Amersham Biosciences) in 100 mM NaCl, 20 mM Tris, pH 7.5, 0.5 mM EDTA, 1 mM β-mercaptoethanol, and 1 mM dithiothreitol.

All the mutated proteins that were purified on the cGMP affinity column migrated on the gel with essentially the same mobility as that of the wild-type GAF domain. Thus, we assume that these single nucleotide changes did not substantially affect transcription, translation, or folding of the protein and that the cGMP binding affinity changes were not due to global effects on protein folding.

Mutant GAF proteins that did not bind to the cGMP affinity column were purified using Talon metal affinity resin (Clontech) using the C-terminal His_6 tag of the protein. Cells were disrupted as above, and the supernatant incubated for 1 h at 4 °C with Talon resin (5 ml of resin/liter of culture) pre-washed three times at 700 × *g* in wash buffer (100 mM NaCl and 20 mM Tris, pH 7.5). After incubation, the resin was washed three times, transferred to a 20-ml Econo-Pac column (Bio-Rad), and washed again with 10 bed-volumes of wash buffer. The resin was then washed once with 10 mM imidazole, and protein was eluted with 150 mM imidazole in 100 mM NaCl and 20 mM Tris, pH 7.5, followed by gel filtration on Superose-12. These mutations also probably do not cause a large global structural change to the protein, because the mutants were expressed in soluble form and purified as a single symmetrical peak of the correct Stoke's radius over the Superose-12 sizing columns. In addition, when three of the non-binding GAF-A/B+C constructs (A459S, K516A, and F522A) were made and tested, all had high catalytic activity, again suggesting a lack of a large global change in the protein. More localized domain changes can not, however, be entirely ruled out without determining the crystal structure of each mutant protein.

Bovine PDE2A1 holoenzyme was expressed in Sf9 cells infected with baculovirus (33). Sf9 cells were grown at 27 °C in complete Grace's insect medium (Invitrogen) with 10% fetal bovine serum and 50 μg/ml penicillin/streptomycin in spinner flasks (80–90 rpm) and were infected with 100 ml of virus (at a multiplicity of infection of 10) per liter of media. At 72 h, the cells were harvested and disrupted by microfluidization (5,000 p.s.i.) and then centrifuged at 16,000 × *g* for 15 min. The holoenzyme was purified on an epoxy-Sepharose cGMP affinity column in the presence of 5 mM EDTA followed by gel filtration on Superose-12 (32).

cGMP Competition Binding Assay—To measure cGMP binding, nitrocellulose filter binding assays were conducted in a total volume of 10 ml containing 5 mM Tris, pH 7.5, 25 mM NaCl, 2 mM EDTA, 10 μg/ml bovine serum albumin, and 0.5 nM to 100 μM [8-^{3}H]cGMP (100 μl of ~6000 cpm/pmol [8-^{3}H]cGMP). The reaction was initiated by addition of GAF protein. Dilutions were made until the concentration of binding protein was always at least 3-fold less than the determined IC_{50} value. Following 1 h of incubation on ice, ammonium sulfate was added to a final concentration of 1 M. This mixture was filtered through pre-moistened Millipore HAWP filters (pore size, 0.45 μm) and rinsed twice with a total of 6 ml of ice-cold 1 M ammonium sulfate buffer (20 mM Tris, pH 7.5, 100 mM NaCl, and 5 mM EDTA). In pilot experiments one molar ammonium sulfate was found to be sufficient to maximize binding to each of the wild-type expressed proteins. The filters were dissolved in Filter Count® (Packard) scintillation mixture and counted with a Packard 1600 TR liquid scintillation analyzer. The bound protein counts were corrected by subtraction of nonspecific binding, which was defined as the [8-^{3}H]cGMP bound in the absence of GAF protein. The data were subjected to non-linear least squares analysis using Prism (GraphPad Software) to obtain IC_{50} values.

Other Methods—Total protein concentrations were determined by the method of Bradford, using bovine serum albumin as the standard (Pierce).

RESULTS

Phe-438, Asp-439, and Thr-488 Determine Cyclic Nucleotide Discrimination—We reasoned that one or more of the residues that interact with or are near the positions where the structure of cAMP and cGMP are different should control cyclic nucleotide specificity. Constructs containing mutations of these resi-

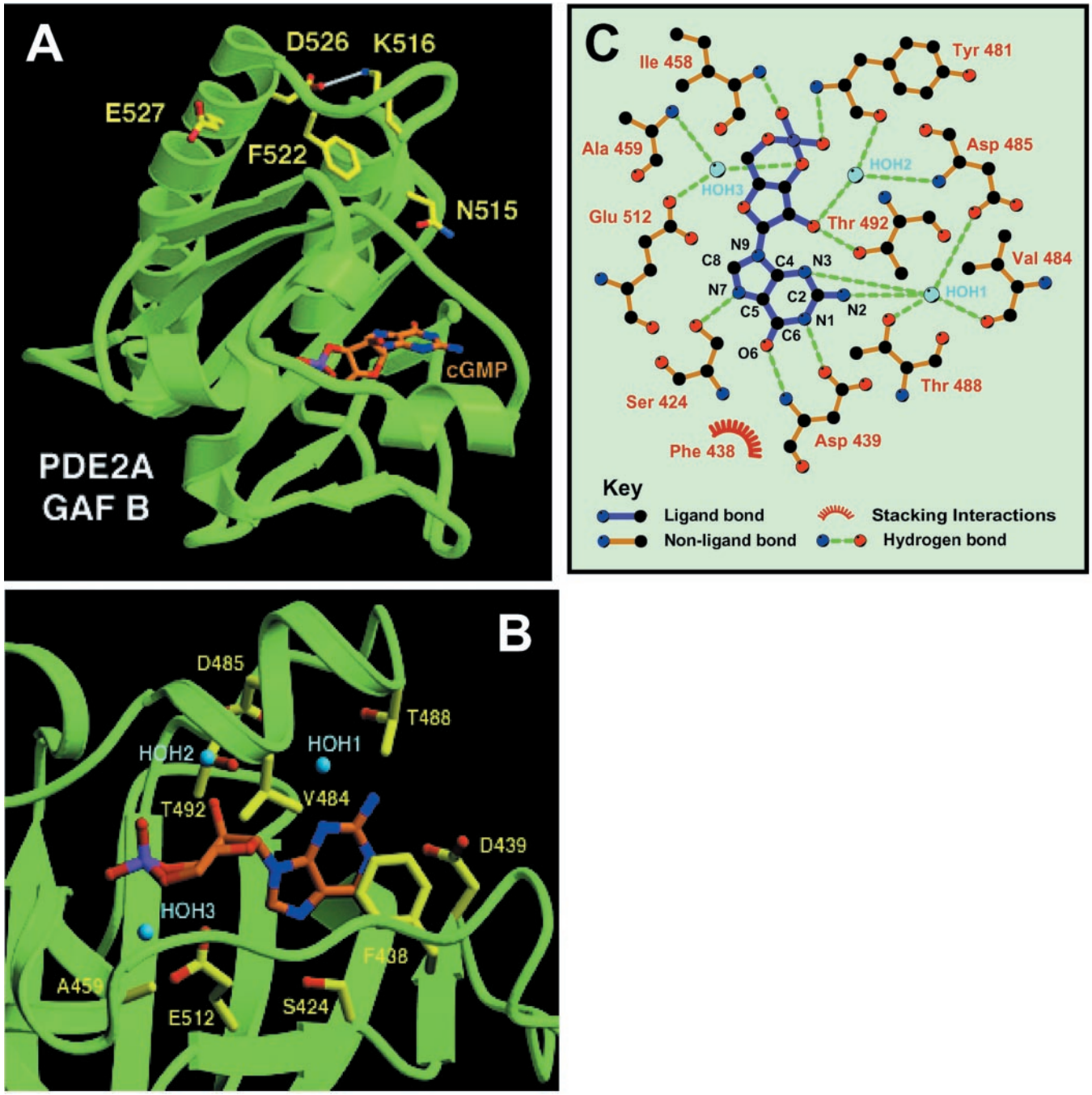

FIG. 2. *A*, PDE2A GAF-B with cGMP bound. The conserved residues of the NKFDE motif are *highlighted in yellow*. Note that these residues are not in the cGMP binding pocket. The lysine (*K516*) and aspartate (*D526*) form a salt bridge (*white dotted line*) that is required for binding. cGMP is *highlighted in orange*. The figure was constructed using MOLSCRIPT® and RASTER3D. *B*, close-up view of the cGMP binding site in PDE2A GAF-B. Shown are nine residues that interact with cGMP in the binding site: six make hydrogen bonds, one base stacks with the guanine ring, and two make backbone amide contacts. This figure was made with MOLSCRIPT and RASTER3D. *C*, close interactions between PDE2A GAF-B and cGMP. The residues interacting with cGMP or HOH are shown. The figure was constructed using LIGPLOT. Carbon, nitrogen, and oxygen atoms are shown in *black*, *blue*, and *red*, respectively. Likely hydrogen bonds are shown by the *dotted green lines*.

dues in PDE2 GAF-A/B were expressed in bacteria and purified using a cGMP affinity column (Fig. 3). The fact that all could bind to and be eluted from the affinity column strongly suggests that each could fold properly. To analyze the effects on substrate selectivity, full IC_{50} curves for both cGMP and cAMP were determined for the wild-type and each mutant. The change in substrate selectivity caused by each mutation is shown by the ratio of IC_{50} values for cGMP and cAMP (Table II). F438A, D439A, and T488A each increased the IC_{50} for cGMP about 2-fold relative to wild-type. However, these mutations each decreased the IC_{50} for cAMP by as much as 8-fold compared with wild-type (Fig. 4). Therefore, the selectivity ratio changes by 16-fold. The increased affinity for cAMP also indicates that these mutations were not generally deleterious to either the overall structure or to the binding pocket of PDE2A GAF-B. Furthermore, the results indicate that a major effect of each mutation is to remove a negative determinant for cAMP binding. Thus, those residues that determine specificity within the binding site appear mostly to restrict the access of cAMP rather than increase the affinity for cGMP.

Asp-439 was replaced with an uncharged asparagine to determine if the carbonyl of the side chain of D439N could form a

TABLE I
Primers used in mutagenesis reactions

Residue change	Primer direction	PDE2A primer sequence
S424A	Forward	5′-GCA GAG ATC TGC GCA GTG TTC CTG CTG-3′
S424A	Reverse	5′-CAG CAG GAA CAC TGC GCA GAT CTC TGC-3′
F438A	Forward	5′-GTG GCC AAG GTG GCC GAT GGT GGC GTT GTG-3′
F438A	Reverse	5′-CAC AAC GCC ACC ATC GGC CAC CTT GGC CAC-3′
F438D	Forward	5′-GTG GCC AAG GTG GAC GAT GGT GGC GTT GTG-3′
F438D	Reverse	5′-CAC AAC GCC ACC ATC GTC CAC CTT GGC CAC-3′
F438E	Forward	5′-GTG GCC AAG GTG GAG GAT GGT GGC GTT GTG-3′
F438E	Reverse	5′-CAC AAC GCC ACC ATC CTC CAC CTT GGC CAC-3′
F438Q	Forward	5′-GTG GCC AAG GTG CAG GAT GGT GGC GTT GTG-3′
F438Q	Reverse	5′-CAC AAC GCC ACC ATC CTG CAC CTT GGC CAC-3′
F438Y	Forward	5′-GTG GCC AAG GTG TAC GAT GGT GGC GTT GTG-3′
F438Y	Reverse	5′-CAC AAC GCC ACC ATC GTA CAC CTT GGC CAC-3′
D439A	Forward	5′-GCC AAG GTG TTC GCT GGT GGC GTT GTG-3′
D439A	Reverse	5′-CAC AAC GCC ACC AGC GAA CAC CTT GGC-3′
D439H	Forward	5′-GCC AAG GTG TTC CAT GGT GGC GTT GTG-3′
D439H	Reverse	5′-CAC AAC GCC ACC ATG GAA CAC CTT GGC-3′
D439N	Forward	5′-GCC AAG GTG TTC AAT GGT GGC GTT GTG-3′
D439N	Reverse	5′-CAC AAC GCC ACC ATT GAA CAC CTT GGC-3′
D439P	Forward	5′-GCC AAG GTG TTC CCG GGT GGC GTT GTG-3′
D439P	Reverse	5′-CAC AAC GCC ACC CGG GAA CAC CTT GGC-3′
A459S	Forward	5′-GAC CAA GGC ATC AGC GGC CAC GTG GCG-3′
A459S	Reverse	5′-CGC CAC GTG GCC GCT GAT GCC TTG GTC-3′
A459T	Forward	5′-GAC CAA GGC ATC ACC GGC CAC GTG GCG-3′
A459T	Reverse	5′-CGC CAC GTG GCC GGT GAT GCC TTG GTC-3′
V484T	Forward	5′-CTT TTC TAT CGC GGC ACC GAT GAC AGC ACT G-3′
V484T	Reverse	5′-C AGT GCT GTC ATC GGT GCC GCG ATA GAA AAG-3′
D485A	Forward	5′-C TAT CGC GGC GTA GCG GAC AGC ACT GGC-3′
D485A	Reverse	5′-GCC AGT GCT GTC CGC TAC GCC GCG ATA G-3′
T488A	Forward	5′-GTA GAT GAC AGC GCT GGG TTC CGC ACA CGC-3′
T488A	Reverse	5′-GCG TGT GCG GAA CCC AGC GCT GTC ATC TAC-3′
T492A	Forward	5′-GC ACT GGG TTC CGC GCG CGC AAC ATT CTC-3′
T492A	Reverse	5′-GAG AAT GTT GCG CGC GCG GAA CCC AGT GC-3′
E512A	Forward	5′-GTC ATT GGT GTG GCT GCG CTA GTG AAC AAG-3′
E512A	Reverse	5′-CTT GTT CAC TAG CGC AGC CAC ACC AAT GAC-3′
N515A	Forward	5′-GCT GAG CTA GTG GCC AAG ATC AAT GGG-3′
N515A	Reverse	5′-CCC ATT GAT CTT GGC CAC TAG CTC AGC-3′
K516A	Forward	5′-GAG CTA GTG AAC GCG ATC AAT GGG CCA TGG-3′
K516A	Reverse	5′-CCA TGG CCC ATT GAT CGC GTT CAC TAG CTC-3′
K516D	Forward	5′-GAG CTA GTG AAC GAT ATC AAT GGG CCA TGG-3′
K516D	Reverse	5′-CCA TGG CCC ATT GAT ATC GTT CAC TAG CTC-3′
F522A	Forward	5′-C AAT GGG CCA TGG GCT AGC AAG TTT GAT G-3′
F522A	Reverse	5′-C ATC AAA CTT GCT AGC CCA TGG CCC ATT G-3′
D526A	Forward	5′-GG TTC AGC AAG TTT GCT GAG GAC CTG GCC-3′
D526A	Reverse	5′-GGC CAG GTC CTC AGC AAA CTT GCT GAA CC-3′
D526K	Forward	5′-GG TTC AGC AAG TTT AAA GAG GAC CTG GCC-3′
D526K	Reverse	5′-GGC CAG GTC CTC TTT AAA CTT GCT GAA CC-3′
E527A	Forward	5′-C AGC AAG TTT GAT GCC GAC CTG GCC ACA G-3′
E527A	Reverse	5′-C TGT GGC CAG GTC GGC ATC AAA CTT GCT G-3′

similar hydrogen bond to that of Asp-439. We also wanted to test if the amino group of the side chain could hydrogen bond to the deprotonated N-1 of cAMP and increase affinity. D439N has a similar affinity (28 ± 11 nM) for cGMP as wild-type (25 ± 2 nM) (Table II). This shows that a negatively charged side chain is not necessary for cGMP specificity. A hydrogen bond between the Asp-439 or D439N side chain and cGMP may still be necessary, because D439A has a slightly lower affinity for cGMP (61 ± 3 nM) than wild-type. As predicted, D439N does increase the affinity for cAMP (131 ± 27 nM) *versus* wild-type (247 ± 14 nM). However, a partial steric clash also is suggested by the fact that D439A has an apparent affinity of 46 ± 13 nM, which is higher than wild-type (247 ± 14 nM) or D439N (131 ± 27 nM). Therefore, the presence of Asp-439 allows for high affinity cGMP binding, but either the aspartate or asparagine side chain antagonizes cAMP binding relative to alanine. A mutation to proline of Asp-439 increases the IC_{50} for cGMP (152 ± 31 nM) to the same levels as cAMP (158 ± 32 nM), which was slightly lower than for wild-type.

Charged, polar, or aromatic substitutions of Phe-438 (F438D, F438E, F438Q, and F438Y) as well as D439H in PDE2A GAF-B (Table III) also gave IC_{50} values that showed weaker cGMP affinity but enhanced cAMP affinity. As with PDE2A GAF-A/B, these GAF-B mutants expressed at comparable levels, bound to a cGMP affinity column, and had an identical mobility on SDS-PAGE as the wild-type protein, suggesting that they were correctly folded, full-length, and capable of binding cGMP. The fact that non-alanine substitutions of Phe-438 and Asp-439 gave similar affinities as the alanine mutations argues that both the phenylalanine and aspartate residues are unfavorable for cAMP binding, rather than enhancing for the binding of GMP.

Effect of Double and Triple PDE2A GAF-B Mutants on Nucleotide Specificity—The previous data suggest that all the residues that interact with the pyrimidine ring are required for cyclic nucleotide discrimination and that loss of any one residue is sufficient to lose specificity. To investigate the hypothesis that combinations of changes to the Phe-438, Asp-439, and Thr-488 binding sites might alter cAMP/cGMP specificities in a manner that even reversed selectivity, a number of double and

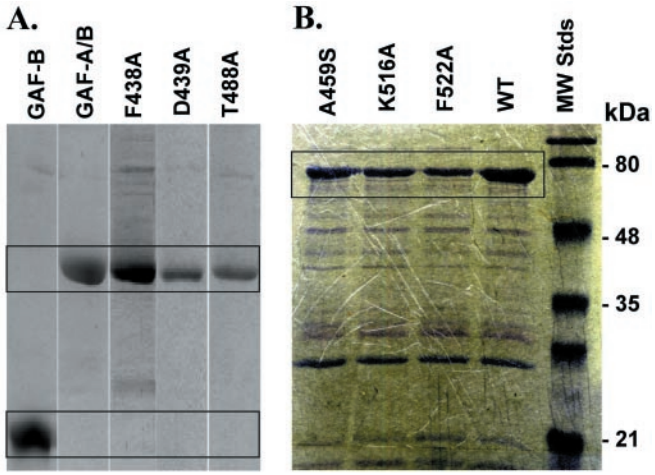

FIG. 3. **SDS-PAGE of the expressed wild-type and mutant PDE2A GAF-B, GAF-A/B, and GAF-A/B+C used in these studies.** Pooled samples (1–3 μg) from wild-type or mutant enzymes, purified through the Superose® 12 column as described under "Experimental Procedures," were applied to each lane. Proteins were visualized with Coomassie Brilliant Blue staining dye. The molecular masses in kilodaltons of protein standards (Bio-Rad) are indicated on the *right*. *A*, *lanes 1–4*, PDE2 GAF-B; *lane 1*, wild-type; *lane 2*, wild-type PDE2 GAF-A/B; *lane 3*, F438A mutant; *lane 4*, D439A mutant; *lane 5*, T488A mutant. *B*, *lanes 1–4*, GAF-A/B+C; *lane 1*, A459S mutant; *lane 2*, K516A mutant; *lane 3*, F522A mutant; *lane 4*, wild-type; *lane 5*, molecular weight standards. *Rectangles* outline the GAF-B, GAF-A/B, and GAF-A/B+C bands.

triple mutants were created in PDE2A GAF-B. F438D, F438E, and D439H substitutions were used in some of the double and triple mutants, because their single mutations gave slightly larger cAMP/cGMP ratio shifts than the alanine mutations. All the double and triple mutants had IC_{50} values similar to the individual single mutations (Table III), and the cAMP and cGMP competition curves become nearly superimposable. Thus, the effect of the double and triple mutants at the binding site appears to mimic the changes in binding caused by any of the single mutations to Phe-438, Asp-439, or Thr-488.

Phosphate-ribose Moiety and Imidazole Ring Contacts Are Critical for cGMP Binding—The crystal structure of PDE2A GAF-A/B with cGMP bound shows the residues that define the binding pocket (Fig. 2*B*) (9). The relative importance of those amino acids that make close contact with the cyclic nucleotide in PDE2A GAF-A/B were investigated by mutating each to alanine and the affinity and specificity of the resultant proteins for both cAMP and cGMP were measured (Table II). Most of these mutant proteins could be isolated on a cGMP affinity column, and therefore were presumably folded correctly. Those that did not bind to the affinity column were investigated after purification on Talon resin and size exclusion chromatography. Individual alanine substitutions of each residue that interacts with the phosphate-ribose moiety (Ala-459, Val-484, Asp-485, Thr-492, and Glu-512) or imidazole ring (Ser-424) resulted in a decrease of cyclic nucleotide binding to below detectable levels (Table II).

NKFDE Motif Is Required for cGMP Binding—Because the

TABLE II
Summary of cAMP and cGMP binding affinities for various GAF domain mutants

The values are the average of three or four experiments ± S.D. Various concentrations of either unlabeled cGMP or cAMP were used as competitive substrates to inhibit the binding of radiolabeled cGMP to purified mouse PDE2A GAF-A, GAF-A/B, GAF-A/B+C, and bovine holoenzyme, and PDE2A GAF-A/B mutants. Labeled ligand concentration was 1 nM, and protein concentration was 0.6 nM. Data shown are representative of experiments performed three times, and values are mean ± S.E. of triplicate determinations.

Amino acid residue change	IC_{50}		Ratio cA/cG	Binding to cGMP affinity column
	cAMP	cGMP		
	nM			
PDE2A wild-type				
GAF-A	ND	ND		(−)[a]
GAF-B	146 ± 10	7 ± 1	20.9	+++
GAF-A/B	247 ± 14	25 ± 2	9.9	+++
GAF-A/B+C	400 ± 20	20 ± 2	20.0	+++
Holoenzyme	598 ± 47	22 ± 1	27.2	+++
Pyrimidine ring-binding residues (GAF-A/B)				
F438A	82 ± 3	61 ± 12	1.3	++
D439A	46 ± 13	61 ± 3	0.8	++
D439N	131 ± 27	28 ± 11	4.7	++
D439P	158 ± 32	152 ± 31	1.0	++
T488A	30 ± 5	38 ± 2	0.8	+
Phosphate-ribose and imidazole ring-binding residues (GAF-A/B)				
S424A	ND[b]	ND		+
A459S	ND	ND		+
A459T	ND	ND		+
V484T	ND	ND		+
D485A[c]	ND	ND		+
T492A	ND	ND		+
E512A	ND	ND		+
NKFDE motif residues (GAF-A/B)				
N515A	ND	ND		(−)
K516A	ND	ND		(−)
K516D	ND	ND		(−)
F522A	ND	ND		(−)
D526A	ND	ND		(−)
D526K	ND	ND		(−)
E527A	ND	ND		(−)
K516D/D526K	ND	ND		(−)

[a] +++ = >0.3 μg/μl; ++ = 0.1–0.3 μg/μl; + = 0.01–0.1 μg/μl; and (−) = <0.01 μg/μl (protein concentration estimates are based on intensity of SDS-PAGE gels stained with Coomassie Blue, compared to known amounts of BSA standard).
[b] ND, not detectible (indicates the protein sample did not bind detectable levels of cyclic nucleotide).
[c] Asp-485 binds both the guanine ring and the phosphate-ribose moiety.

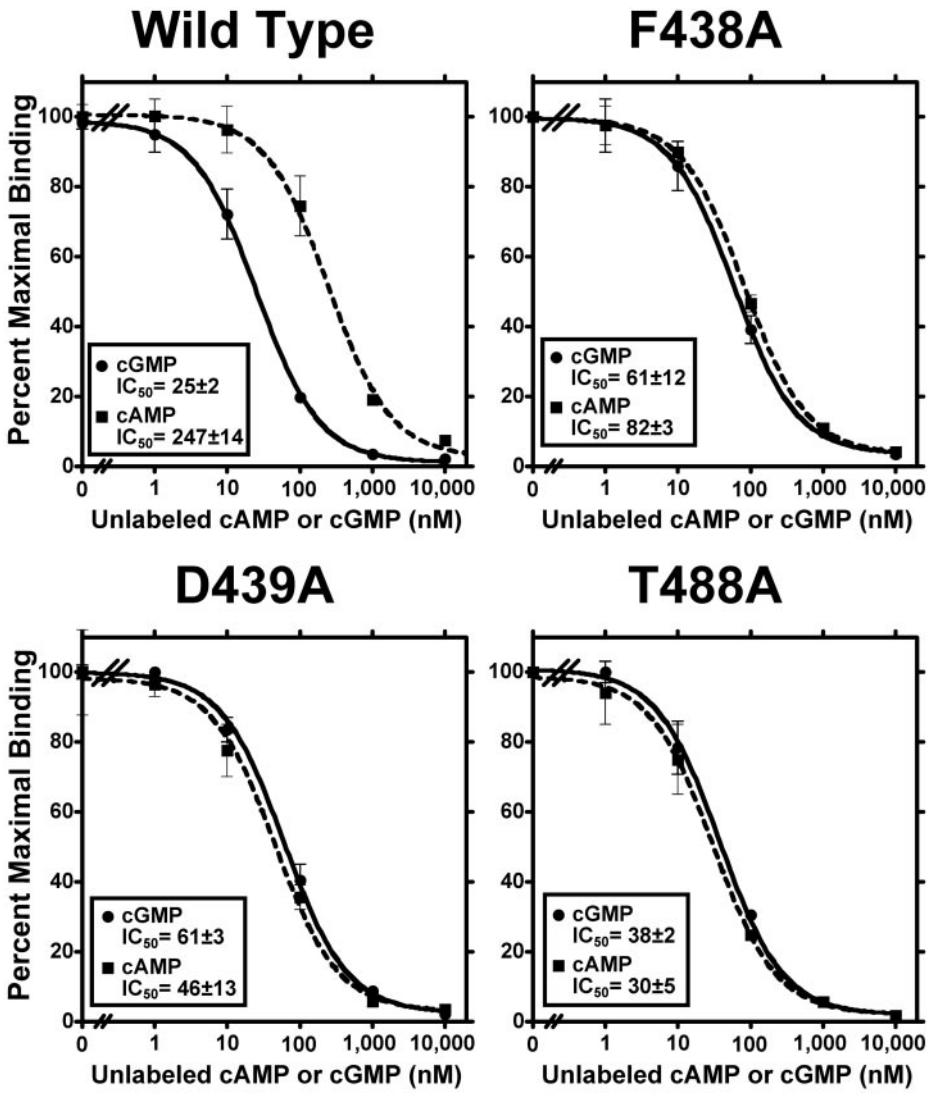

FIG. 4. **Binding analysis for cAMP and cGMP to PDE2A GAF-A/B.** Various concentrations of either unlabeled cGMP (*circles*, *solid lines*) or cAMP (*squares*, *dotted lines*) were used as competitive substrates to inhibit the binding of radiolabeled cGMP to the purified PDE2A GAF-A/B wild-type, F438A, D439A, and T488A. IC_{50} values are given in nanomolar values. Labeled ligand concentration was 1 nM, and the protein concentration was 0.6 nM. The data shown are representative of experiments performed three times, and values are mean ± S.E. of triple determinations.

TABLE III
Binding analysis for non-alanine substitutions and double/triple mutants of PDE2A GAF-B

Various concentrations of either unlabeled cGMP or cAMP were used as competitive substrates to inhibit the binding of radiolabeled cGMP to the purified mouse PDE2A GAF-B wild type and mutants**.** Labeled ligand concentration was 1 nM, and protein concentration was 0.6 nM. Data shown are representative of experiments performed three times, and values are mean ± S.E. of triplicate determinations.

PDE2A GAF	Amino acid residue change	IC_{50}		Ratio cA/cG
		cAMP	cGMP	
		nM		
B	Wild type	146 ± 10	7 ± 1	21
B	F438D	19 ± 3	21 ± 2	0.9
B	F438E	14 ± 3	19 ± 11	0.7
B	F438Q	28 ± 5	25 ± 8	1.1
B	F438Y	21 ± 2	33 ± 7	0.7
B	D439H	61 ± 9	41 ± 2	1.5
B	F438D/D439A	18 ± 3	33 ± 6	0.6
B	F438D/D439N	19 ± 2	24 ± 3	0.8
B	F438E/D439A	19 ± 4	37 ± 8	0.5
B	F438E/D439N	20 ± 5	41 ± 12	0.5
B	F438E/T488A	45 ± 8	34 ± 6	1.3
B	D439H/T488A	33 ± 7	44 ± 7	0.8
B	F438E/D439A/T488A	57 ± 2	37 ± 6	1.5

NKFDE motif residues are conserved within many mammalian PDE and *Anabaena* adenylyl cyclase GAF domains, it has been predicted that this motif is directly involved in binding cyclic nucleotides. However, in the PDE2A crystal structure, the NKFDE motif is found away from the binding pocket (9). Thus, we sought to determine whether these residues play a role in cGMP binding to PDE2A, as has been suggested in PDE5A (13). All five NKFDE residues of PDE2A GAF-B were changed to alanine individually, and the mutants were tested for cGMP binding. Isolation of these proteins required use of the His_6 tags and purification using Talon resin, because none of them bound to the cGMP affinity resin. When tested by filter binding assay, none of these mutant proteins bound cGMP at detectable levels (Table II). To test if the Lys-Asp salt bridge stabilized a conformation that allows cGMP to bind in the binding pocket, three mutants were created: NDFDE (K516D), NKFKE (D526K), and NDFKE (K516D/D526K), where the Lys and Asp residues were switched for each other. None of these three mutants bound cGMP at detectable levels (Table II), suggesting that each prevented the proper formation of the cGMP binding pocket in PDE2. These results differ from the effects of a similar mutation in PDE5 where much smaller decreases in cGMP binding affinity were seen (13).

GAF-A and the Catalytic Domain Alter cGMP Binding Affinity to GAF-B of PDE2A—PDE2A is composed of an N-terminal domain, tandem GAF domains (A and B), and a C-terminal catalytic domain (C) (Fig. 1). As shown in Table II, mouse PDE2A GAF-A/B binds cGMP with an IC_{50} of 25 ± 2 nM, or as much as 40 times lower than previously reported for the bovine

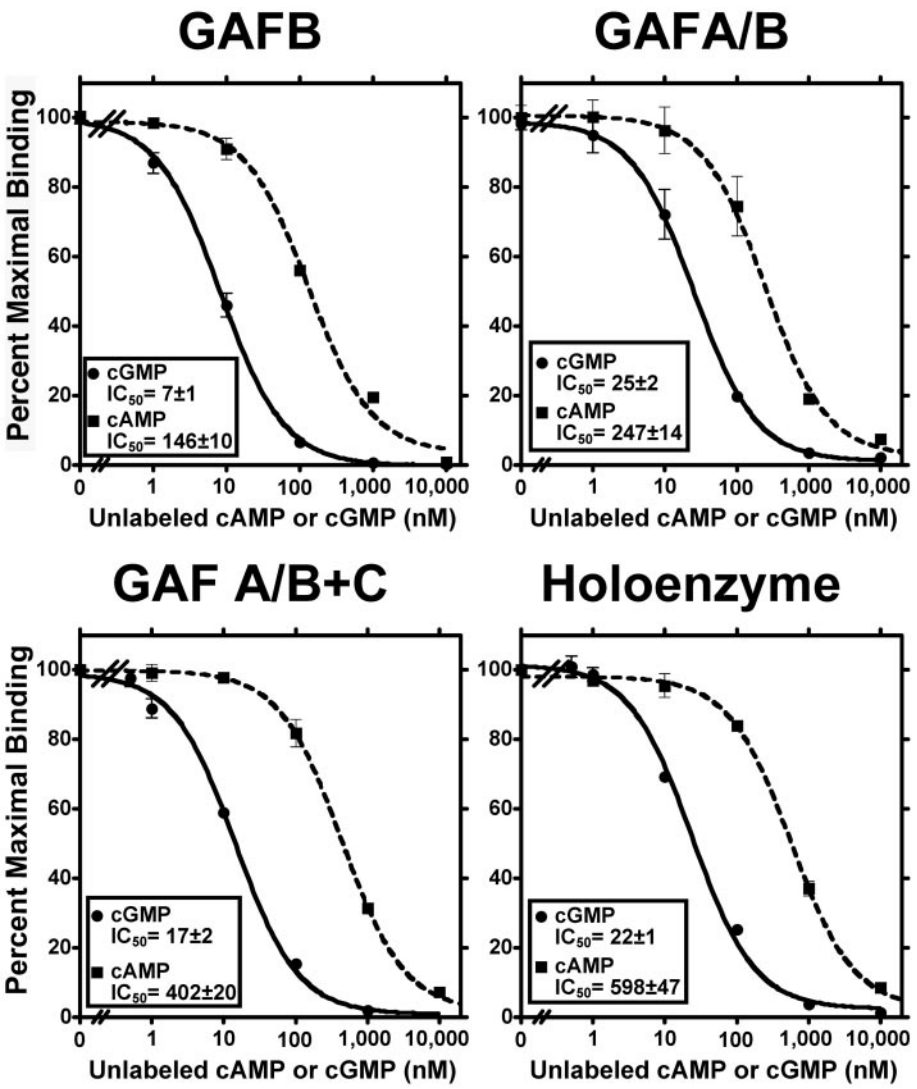

FIG. 5. **Binding analysis for cAMP and cGMP binding to holoenzyme GAF domain proteins.** Various concentrations of either unlabeled cGMP (*circles* and *solid lines*) or cAMP (*squares* and *dotted lines*) were used as competitive substrates to inhibit the binding of radiolabeled cGMP to the purified mouse PDE2A GAF-B, GAF-A/B, GAF-A/B+C, and bovine holoenzyme. IC_{50} values are given in nanomolar values. Labeled ligand concentration was 1 nM, and the protein concentration was 0.6 nM. The data shown are representative of experiments performed three times, and values are mean ± S.E. of triplicate determinations.

PDE2A1 holoenzyme (34, 35). In the holoenzyme, binding of cGMP to the allosteric GAF-B region results in activation of the catalytic domain, as shown by a greater than 10-fold increase in activity (36–39). To test the hypothesis that the presence of the catalytic and N-terminal domains would affect cGMP binding to GAF-B, the holoenzyme and a number of GAF domain proteins were expressed, and binding affinities measured under exactly the same conditions as for the GAF-B domain alone.

Mouse PDE2A, GAF-A, GAF-B, GAF-A/B, and GAF-A/B+C were expressed in bacteria as described under "Experimental Procedures." The catalytic activity of the baculovirus-expressed bovine PDE2A1 holoenzyme (~60 ± 4 μmol/min/mg at 40 μM cGMP substrate) agreed reasonably well with values reported in the literature for pure enzyme isolated from heart or adrenal gland (32). Purified PDE2A GAF-A does not bind either cAMP or cGMP at detectable levels (Table II), which is consistent with the crystal structure that showed cGMP bound to only the GAF-B domain of PDE2A GAF-A/B (9). The cGMP IC_{50} value for GAF-B (7 ± 1 nM) is ~3-fold less than GAF-A/B (25 ± 2 nM), GAF-A/B+C (20 ± 3 nM), or the holoenzyme (22 ± 1 nM) (Fig. 5). The cAMP IC_{50} value for GAF-B (146 ± 10 nM) is almost 2-fold less than GAF-A/B (247 ± 14 nM), 3-fold less than GAF-A/B+C (400 ± 20 nM), and 6-fold less than the holoenzyme (598 ± 47 nM) (Fig. 5). Thus, the presence of GAF-A appears to decrease both cAMP and cGMP affinity, whereas the N terminus and catalytic domain appear to have a further effect to diminish cAMP but not cGMP affinity.

GAF-B Mutations Do Not Greatly Affect Global Stability or Global Folding—It was possible that the effects of some of the mutations described previously were due to effects on the stability or global folding of the expressed proteins. We were particularly worried about the mutations made in the NKFDE region and those in close contact with the ribose moiety as they showed no cGMP binding. Therefore, we developed a GAF-A plus GAF-B plus catalytic domain bacterial expression vector (GAF-A/B+C). This allowed the effects of mutations on more than one functional domain to be measured. Interestingly, the specific catalytic activity (V_{max}) of the bacterially expressed A/B+C wild-type construct was very high, in fact by as much as 4 times higher than the highest activity reported in the literature for any native holoenzyme (2, 6). As shown in Table IV, the A459S, K516A, or F522A mutations on A/B+C abolished high affinity cGMP binding as they did in the isolated GAF domains alone. However, each of these constructs still had high catalytic activity (25–55% of recombinant wild-type A/B+C and >100% of the value reported for holoenzyme purified by classic procedures from bovine heart). Therefore, it appears that the mutations did not cause a global denaturation of the protein.

DISCUSSION

Roles of Phe-438, Asp-439, and Thr-488 in GAF-B of PDE2A—Phe-438, Asp-439, and Thr-488 appear to control nucleotide specificity in the PDE2A GAF-B binding site. The mutation F438A in PDE2A GAF-A/B changed the wild-type

TABLE IV
Specific activity and binding affinities of PDE2A "A/B+C" constructs isolated from the final sizing column

Values are the averages of duplicate experiments plus or minus the range. Corrections for purity were made based on analysis using ImageJ® from Coomassie Blue-stained SDS-PAGE gels.

Construct	PDE activity, pooled peak	PDE activity (corrected for purity)	CGMP binding (IC_{50})
	μmol/min/mg		*nM*
A459S	38.6 ± 7.2	271 ± 68	ND[a]
K516A	18.2 ± 1.1	135 ± 22	ND
F522A	16.6 ± 0.2	132 ± 11	ND
ABC WT	68.1 ± 1.1	404 ± 49	53.2 ± 4.1
Wild-type holoenzyme[b]		120	

[a] ND, not determined.
[b] Value taken from Martins *et al.* (32).

IC_{50} values from 247 ± 14 nM to 82 ± 3 nM for cAMP and from 25 ± 2 nM to 61 ± 12 nM for cGMP. Therefore, the ability of GAF-B to discriminate between cGMP and cAMP clearly relies at least in part on residue Phe-438. The crystal structure of PDE2A GAF-A/B shows that the side chain of Phe-438 is base-stacked with the guanine ring (Fig. 2*B*) (9). Stacking interactions are common in many nucleotide-binding domains. For example, in the crystal structure of glycogen phosphorylase, aromatic amino acids in the binding sites base stack with the adenine rings of AMP and NADH (27). It has been postulated from analog studies that there is a difference in polarization potential of the purine ring of cAMP *versus* cGMP toward the polarizable amino acid side. Because the magnitude and direction of the dipole moment of the purine ring in cGMP is significantly different than in cAMP, the guanine base would be expected to have different dipole-induced dipole interactions with the polarizable Phe-438 (40). These differences have been used to explain catalytic specificity preferences for cyclic nucleotide analogs at the PDE2 catalytic site (39, 40). Actually, because these studies were conducted with PDE2 holoenzyme and we now know that the GAF domains can regulate the activity of the catalytic domain of the enzyme, it may be that the measured catalytic activities were in part due to differences in GAF domain binding of the analogs. Regardless of whether the changes seen with these early studies were due to GAF domain or catalytic domain binding, the changes in IC_{50} values seen in PDE2A GAF-B with the replacement of F438Y suggest such a mechanism may hold. F438Y shows a similar cAMP and cGMP IC_{50} shift as that of the alanine mutation to that site, presumably due to a preferred solvent-stabilized orientation of the polar side chain of tyrosine that does not allow for base-stacking interactions with tyrosine.

D439N contains a side-chain amide group in place of a carboxylate but still retains the ability to hydrogen bond to N-1 of cGMP, likely explaining its similar cGMP affinity as wild-type. The asparagine presumably can still act to exclude cAMP, because its steric character mimics aspartate, unlike alanine, which has a much smaller methyl group side chain. D439P removes the potential for a hydrogen bond to form between the Asp-439 backbone amide group and the guanine ring carbonyl at C-6, which would be expected to decrease cGMP affinity. On the other hand, this mutation also should eliminate a clash with the amino group at C-6 of cAMP allowing for increased cAMP binding.

Alanine mutations of Asp-439 and Thr-488 also remove hydrogen bonding potential for cGMP, likely explaining the ~2-fold increase in IC_{50} for cGMP (Fig. 6, *A* and *B*). The IC_{50} for cAMP is decreased nearly 8-fold by D439A, presumably by removing a polar side-chain clash with the N-1 nitrogen group of cAMP (Fig. 6, *C* and *D*). With more bulk solvent exposure of HOH1, T488A also may reduce this clash by allowing the adenine ring to move away from the Asp-439 backbone amide. In the double and triple mutants of Phe-438, Asp-439, and Thr-488, the Asp-439 side-chain clash would probably be reduced to the same level as with a single mutation. This likely explains why the effects of the individual mutations were not additive. Interestingly, the multiple alanine mutations did not prevent the cyclic nucleotide from being stabilized in the binding pocket and still allowed for high affinity cGMP binding.

PDE2A GAF-B: An Alternate Model for Nucleotide Selectivity—PDE2A GAF-B offers a new model for high affinity guanine nucleotide binding where three residues are all required to interact together to determine cyclic nucleotide selectivity (Fig. 7, *A* and *B*). This differs from other high affinity guanine nucleotide-binding proteins, such as a membrane guanylate cyclase (RetGC-1) and rod cyclic nucleotide-gated ion channels, where a single carboxylate of a glutamate or an aspartate, respectively, interact with both N-1 and N-2 of the guanine ring (Fig. 7, *C* and *D*) (17, 20). In the bovine rod cyclic nucleotide-gated ion channel, the substitution by a non-polar residue of the single aspartate residue in the binding domain significantly inverted nucleotide selectivity in favor of cAMP by removing unfavorable electrostatic interactions with the free electron pair of N-1 (17). This type of interaction between the guanine ring and an aspartate residue in the rod cyclic nucleotide-gated ion channel is identical to that shown in other high affinity GTP-binding proteins such as transducin-α (22), EF-Tu (21), and H-ras p21 (23). Neutralization of the analogous aspartate residue in H-ras p21 (41) and EF-Tu (42) also resulted in decreased nucleotide selectivity. Thus, although a general precedent exists for a role of aspartate or glutamate on nucleotide selectivity, the natures of the interactions vary greatly among different proteins.

In PDE2A GAF-B, Asp-439 also makes a similar hydrogen bond to N-1 with its carboxylate; however, it does not bind to N-2. This allows the other two residues to interact with the guanine ring through a water molecule or base stacking. Thus, Phe-438, Asp-439, and Thr-488 all must be present to achieve the selectivity found in the GAF-B binding site. Furthermore, unlike other cyclic nucleotide binding sites, cGMP is bound in its extended, anti-conformation, and the phosphate group of cGMP is stabilized in part by the dipole of helix α3 (9). Therefore, PDE2A GAF-B offers an alternative model for high affinity guanine nucleotide binding by demonstrating how multiple residues may act in concert to control nucleotide discrimination.

Mechanism of Cyclic Nucleotide Binding—The data presented suggest that the residues contacting the phosphate-ribose moiety are absolutely required for high affinity cGMP binding to the GAF-B domain. Val-484 makes van der Waals contact with the ribose ring, and its replacement with a threonine abolishes cGMP binding. If this side chain rotates 90°, the hydroxyl group from the threonine would be better exposed to solvent, but this is apparently overridden by the fact that the γ-methyl group would then clash with the ribose ring oxygen (Fig. 2*B*). Even mutations to serine or threonine of Ala-459, a residue that makes backbone contacts to the phosphate-ribose moiety, abolish cGMP binding. The Cβ of Ala-459 is buried within the binding pocket and is in close proximity with Glu-512 (Fig. 2, *B* and *C*). Thus, the hydrophilic hydroxyl groups on serine and threonine may disturb packing in the region, displace HOH3, or form hydrogen bonds with the OE1 of Glu-512. Any of these factors individually or in concert may explain the apparent loss of cGMP binding seen in this mutant.

Asp-485 is another binding pocket residue where an alanine mutation abolishes binding. The side chain of Asp-485 forms a 3.2-Å hydrogen bond contact with HOH1 that binds to the

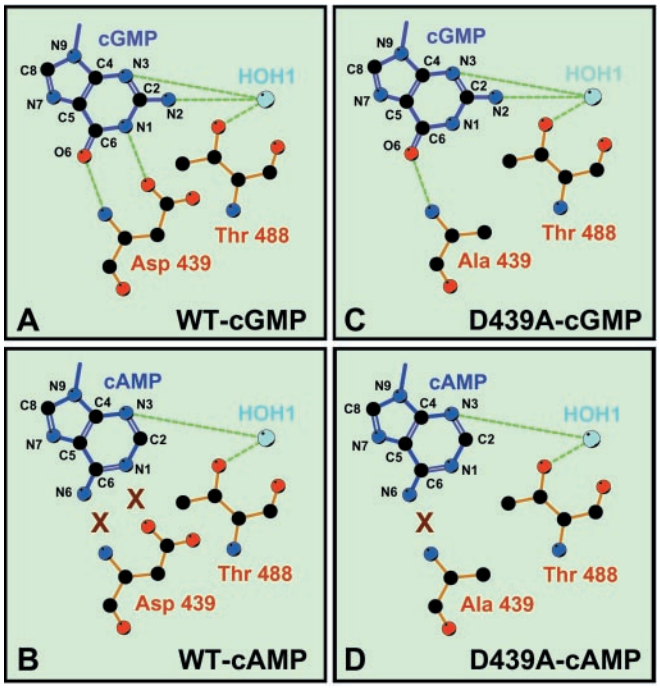

FIG. 6. **D439A mutation removes cyclic nucleotide selectivity of the GAF-B domain in PDE2A.** cGMP and cAMP (the cognate ribose and cyclic phosphate portions are not shown) are modeled into the binding site of PDE2 GAF-B with residues and hydrogen bonding shown (*A* and *C*). Removal of the aspartate side chain (D439A) reduced the favorable interactions with cGMP at N-1 while eliminating one repulsive interaction (*X*) with cAMP (*B* and *D*). Phe-438 (not shown) base stacks with the guanine ring of cGMP.

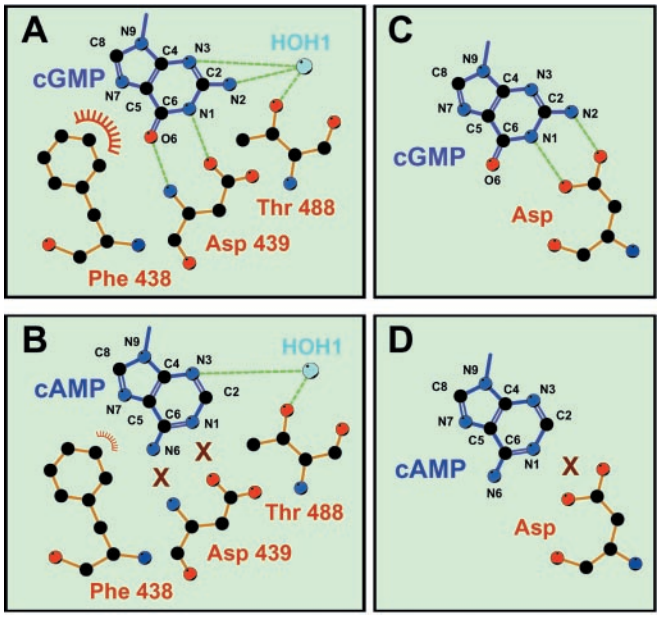

FIG. 7. **Hypothesis for purine selectivity by the GAF-B domain on PDE2A.** cAMP and cGMP (cognate ribose and cyclic phosphate portions not shown) are modeled into the binding site of PDE2 GAF-B. The difference in affinity between cGMP and cAMP binding to PDE2A GAF-B appears due to three positive interactions (N-1, N-2, and O-6) with cGMP (*A*) and two negative interactions (N-1 and N-6) with cAMP (*B*), as well as differences in stacking interactions with Phe-438. This differs from other high affinity guanine nucleotide-binding proteins, where a carboxylate group bonds with the N-1 and N-2 of cGMP (*C*) while it is repulsed by the free electron pair of N-1 in cAMP (*D*).

pyrimidine ring and forms a van der Waals contact with HOH2 that binds to the ribose ring (9). The backbone amide of Asp-485 forms a 3.0-Å hydrogen bond contact also with HOH2 that binds to the ribose ring (Fig. 2*C*) (9). Thus, D485A removes the side chain and van der Waals contacts, which would likely perturb the hydrogen bonds mediated by the two waters and cause the loss of cGMP binding seen in this mutant.

The complete loss of cGMP binding observed for mutations at residues that interact with the phosphate-ribose moiety of cGMP suggests that the majority of the energetic contribution for binding may be attributed to the phosphate-ribose ring-binding residues, which stabilize the nucleotide-bound form of the enzyme. In studies on the bovine rod cyclic nucleotide-gated ion channel, the closed channel also has been suggested to bind the cyclic nucleotide primarily through its ribose and phosphate (17). In this channel, the purine ring of the cyclic nucleotide does not appear to be important for initial binding to the channel, but rather its interactions with the side chain of an aspartate in the binding site appear to control the allosteric conformational change leading to channel opening (17). Thus, a significant portion of the binding energy residing within the bonds formed between the nucleotide ribose and phosphate and the binding pocket residues may be a widespread mechanism by which many high affinity guanine nucleotide-binding proteins stabilize cyclic nucleotides within their binding sites. It was surprising that a single alanine mutation within the binding pocket could abolish binding. These mutations may have affected the local folding of the protein, or perhaps disturbed a network of hydrogen bonds making the protein sensitive to the removal of one of them. Such hydrogen bond networks that are sensitive to single changes have been discussed previously (43, 44).

NKFDE Motif May Link GAF-B and the Catalytic Domain—Initially it was a great surprise to find that none of the NKFDE residues of GAF-B are in contact with cGMP, because they were conserved in most cGMP-binding PDEs and because mutation of the NKFDE reduced binding in PDE5. Therefore, it was important to determine the effects of mutations to these residues in PDE2. Again, somewhat unexpectedly, mutations to these residues completely abolished cGMP binding. From the crystal structure, it seemed possible that mutations of the NKFDE motif may disrupt interactions between Glu-512 and cGMP, as Glu-512 is near Asn-515, at the beginning of the motif. Glu-512 is a critical residue in the binding pocket (Table II). It also seems possible that the substitutions NDFDE (K516D) and NKFKE (D526K) would disrupt the salt bridge within the NKFDE motif and that this bridge is required for cyclic nucleotide binding (Fig. 2*A*). Moreover, the NDFKE (K516D/D526K) result further suggests that the order of the residues, Lys followed by Asp, is essential. It remains a possibility that the lack of binding seen in the NKFDE mutants is because this motif is essential for correct folding and nothing else. However, the data with the A/B+C construct would argue against this possibility. Because the catalytic activity of these constructs remained largely intact, the mutations clearly did not introduce a large global change in the protein. The fact that the specific activity is higher than the wild-type may suggest that some part of the molecule, presumably the N-terminal region, normally inhibits catalytic activity. However, this hypothesis will take further experimentation to test rigorously. Why the NKFDE motif is so well conserved and absolutely required for binding in PDE2A clearly deserves further investigation but may require crystallization to solve.

Differential Effects of cAMP and cGMP—Evidence in the literature suggests that, in PDE2A, cAMP and cGMP induce different conformations. For example, chemical modification of PDE2A with dicyclohexylcarbodiimide partially activates the enzyme but only in the presence of cGMP, not cAMP, suggesting that the cGMP-induced conformational changes are differ-

ent from cAMP-induced changes (6). In earlier studies, the cGMP-bound enzyme was shown to be more susceptible to chymotrypsin inactivation than the cAMP-bound enzyme (6, 45). The major chymotrypic cleavage site exposed by cGMP binding is at tyrosine 553 (6), which is found in the α5 helix of GAF-B, implying that this region takes part in the conformational change. These findings further suggest that cGMP binding to the GAF-B domain creates a ligand-dependent conformational change that is transmitted mechanically, to expose the α5 helix to chymotrypsin inactivation, or the enzyme to dicyclohexylcarbodiimide modification and activation. A similar mechanism of activation has been suggested to occur in the guanine nucleotide exchange protein directly activated by cAMP (Epac), where binding of cAMP transmits a conformational change via a "hinge" to induce a structural change in the C-terminal portion of the protein (46). Taken together, these findings support the notion that both cAMP and cGMP bind GAF-B but transmit distinct conformational changes to the catalytic part of the enzyme. Therefore, it was not too surprising in the current studies that specific point mutations could differentially alter cAMP and cGMP binding affinity.

High Affinity of PDE2A—An initially surprising finding was that the individual GAF domain constructs bound cGMP with very high affinity, in fact much higher than initially reported for the PDE2 holoenzyme. It seemed possible that microscopic reversibility might in part explain the decrease in binding affinity in the holoenzyme due to the presence of the catalytic domain, because cGMP binding to GAF-B and activation of the catalytic domain are coupled. A tighter cyclic nucleotide binding affinity in individual GAF domains relative to the tandem GAF domains or holoenzyme have also been reported recently in PDE5A (16). Thus, neighboring domains may weaken the binding affinity in other mammalian PDE GAF domains. In the present studies, the neighboring GAF-A and catalytic domains appear to have an additive affect on the IC_{50} values for cAMP but not cGMP binding to GAF-B (Table II). Again, the complete explanation for these differences will probably require a crystal structure for the holoenzyme in the bound and unbound state.

The apparent cAMP and cGMP binding affinities reported here for all the PDE2A GAF domain proteins, including the holoenzyme, are substantially higher than those previously reported (6, 32, 34, 36, 47). This is likely, because the assays performed in this study used very low protein concentrations (~1 nM). In order for the IC_{50} value to approach K_i, the level of protein in the assay must be substantially less than the K_d of the radioligand.[2] Most if not all of the previously reported cAMP and cGMP binding affinity studies have been performed at much higher protein concentrations or used cell extracts where the exact enzyme concentration was unknown (6, 32, 34, 36, 47). In retrospect, it can be calculated that nearly all of these earlier studies did not meet the normal Michaelis-Menten assumption that the concentration of the ligand be much higher than the binding protein. Therefore, for high affinity binding sites, this would have resulted in an apparent titration until ligand levels started to exceed protein concentration. Experimentally, in the present studies when the protein concentration was decreased, lower IC_{50} values for cGMP for the wild-type enzyme were observed. Thus, previously reported cGMP affinities for PDE2A (6, 32, 34, 36, 47) are likely to be artificially low due to this effect. Another formal possibility, however, is that the higher dilutions used in the current studies cause a dilution-induced disaggregation of PDE2 to a form that has a higher intrinsic binding affinity.

Regardless of the reason, the question arises as to the possible physiological consequences of such high affinity sites. In many tissues with high local concentrations of PDE2A, such as brain, adrenal gland, and olfactory cilia, enzyme levels are likely to be quite high, well above "basal" cGMP concentrations (5, 49, 50). Under these conditions, where the GAF domains would not be fully occupied, the high affinity binding site on PDE2A would be expected to function as a fast, sensitive, "on" switch for PDE2 when cGMP is increased. The off-rate of the switch would then be determined by the off-rate of the nucleotide from the GAF domain.

Examples of very rapid cyclic nucleotide changes have been seen in olfactory neurons (51), astrocytes and strial cells (52), platelets (53, 54), and the heart (55), all of which are known to contain high levels of PDE2A. Such a fast response to cGMP could clearly be related to the rapid activation of PDE2 caused by cGMP binding to the GAF-B domain. If the GAF domains are as exquisitely sensitive to cyclic nucleotide concentrations in intact tissues as in these *in vitro* studies and no other factors in the cell are identified that modulate cGMP affinity, we may need to re-evaluate our ideas about regulation of this enzyme in physiological situations.

Finally, these studies raise questions about the mechanisms by which cyclic nucleotide binding to the GAF-B domain is allosterically coupled to changes in activity at the catalytic site. This is especially true, because the concentrations of cyclic GMP that allosterically activate the holoenzyme *in vitro* are much higher than the cGMP affinity constants determined in this study. A complete analysis of the effects of the mutations described in the present study on the PDE2 holoenzyme as well as mutations at the dimerization interfaces and likely in the catalytic domain will probably be needed to fully address this problem. Ultimately, it likely will require solution of the structure of the holoenzyme to know where to begin these studies.

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[2] To obtain an estimate of the affinity of the unlabeled ligand (K_i), the Cheng-Prusoff relationship may be applied (48): $K_i = IC_{50}/(1 + [L]/K_d)$. In this equation, the IC_{50} value of a competitive inhibitor is related to the K_i value of the inhibitor as a function of the labeled ligand concentration ($[L]$) and the K_d value for the labeled ligand. In all the experiments, the labeled ligand concentration, $[L]$, was 1 nM labeled cGMP, and the lowest measured K_d for all the proteins assayed was 7 nM. Thus, the denominator would in all cases be very close to 1, and the IC_{50} value will closely approximate K_i.

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